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Research Note

Inactivation of *Bacillus cereus* Spores on Stainless Steel by Combined Superheated Steam and UV-C Irradiation Treatment

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ABSTRACT

Bacillus cereus spore contamination on food contact surfaces is of great concern in the food industry. Thus, in the present study, superheated steam (SHS) was used alone or combined with UV-C irradiation for inactivation of *B. cereus* spores inoculated on stainless steel coupons. Temperatures higher than 250°C were needed to effectively inactivate *B. cereus* spores by SHS treatment alone, while a synergistic bactericidal effect resulted from the sequential treatment of SHS before or after UV-C irradiation. The increased dipicolinic acid ratio obtained by the combined treatment had a significant role in the synergistic bactericidal effect. Therefore, the combined treatment of SHS and UV-C could be used effectively to inactivate *B. cereus* on stainless steel. It is recommended to use hurdle technology with reduced energy consumption to ensure microbiological safety on food contact surfaces.

HIGHLIGHTS

- Inactivation of *Bacillus cereus* spores on stainless steel was identified in this study.
- Superheated steam (SHS) was applied solely or combined with UV-C irradiation.
- A synergistic effect was observed by combination treatment for spore inactivation.
- The dipicolinic acid (DPA) release level increased significantly by combination treatment.
- The combination treatment can be applied to sanitize food processing equipment.

Key words: *Bacillus cereus* spore; Hurdle technology; Stainless steel; Superheated steam; Synergistic effect; UV-C irradiation

Contamination by *Bacillus cereus* on food contact surfaces is a great concern not only because of biofilm formation but also because of the sporulation of the pathogen. It is well known that the resistance of bacterial pathogens increases significantly by sporulation, and the structure of spores has an important role in increased resistance *(6)*. Spore structure consists of the inner membrane, which exists between the core and cortex, and the outer membrane, which exists between the cortex and coat. Pyridine-2,6-dicarboxylic acid, also called dipicolinic acid (DPA), is located in the core of the endospore and is a chelate with divalent cations, mostly Ca^{2+} *(19, 25)*. DPA can be released when germination occurs or the inner membrane of the endospore ruptures *(12, 18)* and is usually followed by the loss of resistance and a change in the germination rate *(3, 15)*. This robust structure of endospores and the presence of DPA make it difficult to inactivate *B. cereus* spores on surfaces. Moreover, note that sporulation of *B. cereus* can occur during biofilm formation *(17)*.

Therefore, a high level of bactericidal treatment is required to inactivate *B. cereus* spores due to their various resistance against heat, radiation, and chemical stress. Superheated steam (SHS), defined as steam that is heated to a temperature above the boiling point by secondary heating of saturated steam at a given pressure, has demonstrated its microbicidal effects *(14)*. When the surface is exposed to SHS, the SHS condenses and transfers a large amount of heat, including latent heat *(8)*. After the surface is heated, the condensate evaporates rapidly over a saturation point, leaving no water droplets *(9)*. Steam has been known as a safe and environmentally friendly technology, and Ban et al. *(1)* indicated that SHS was more effective in inactivating biofilm cells than was saturated steam on polyvinyl chloride and stainless steel. In this regard, SHS was used to inactivate *B. cereus* spores on stainless steel. Another technology to inactivate pathogens on the surface is UV-C irradiation by using a low-pressure Hg lamp. UV-C radiation at 254 nm induces the formation of 6-4 photoproducts or cyclobutane pyrimidine dimers in vegeta-

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tive cells and 5-thyminyl-5,6-dihydrothymine in endospores, which can cause cell death *(11, 20, 22)*. UV-C irradiation does not generate toxic compounds and has been known to have synergistic microbicidal effects when combined with mild heat treatment *(4)*.

Ensuring microbiological safety on stainless steel, which is widely used in food processing facilities, is of great importance in the food industry. However, studies on the inactivation of *B. cereus* spores on stainless steel by SHS and its combination with UV-C irradiation are limited. Thus, in the present study, *B. cereus* spore inactivation by SHS, UV-C, and a combined treatment were investigated and confirmed with a DPA release experiment.

MATERIALS AND METHODS

Spore preparation and inoculation. Three strains of *B. cereus* (ATCC 10876, ATCC 13061, and ATCC 14579) were obtained from the bacterial culture collection of the School of Food Science, Seoul National University (South Korea). Each strain (maintained in –80°C frozen stocks) was streaked for isolation onto tryptic soy agar (TSA; Difco, BD, Sparks, MD) and incubated for 24 h at 37°C. A single colony of each strain was inoculated into 5 mL of tryptic soy broth (Difco) and incubated for 24 h at 37°C. For spore formation, 1 mL of overnight culture of each strain and 9 mL of 0.2% peptone water (PW; Difco) were spread onto three TSA plates, which were then incubated for 5 days at 30°C. Agar surfaces were rubbed with a sterile swab to collect cell suspensions. The cell suspensions were mixed, collected by centrifugation at 4,000 × *g* for 20 min at 4°C, and washed three times with 0.2% PW. The final pellets were resuspended in sterile 0.2% PW, corresponding to approximately 10^9 to 10^{10} CFU/mL. Type 304 stainless steel coupons with no. 4 finish were immersed in 95% ethanol for 60 min to disinfect the surface and then flame sterilized. Nine drops (50 μL of cell suspension) were spot inoculated onto the coupon surface and dried with a fan for 30 min.

Bactericidal treatments. The SHS system consisted of a primary heater and secondary heater. Each inoculated stainless steel coupon was subjected to 200, 250, and 300°C SHS for a maximum treatment time of 5 min. For the combination treatment, SHS was administered sequentially with 15, 30, and 60 min of UV-C radiation. SHS used before or after UV-C irradiation was noted as SHS+UV-C or UV-C+SHS, respectively, to identify the effect of treatment sequence. A synergistic effect was identified by comparing the sum of reduction levels by each treatment with those of combined treatments.

Bacterial enumeration. After treatment, coupons were deposited in sterile 50-mL conical centrifuge tubes containing 20 mL of PW and 2 g of sterile glass beads (425 to 600 μm; Sigma-Aldrich, St. Louis, MO) and then agitated with a benchtop vortex mixer set at maximum speed for 1 min. After detachment, each tube was submerged in an 80°C water bath (BW-05G; Jeio Tech, Daejeon, South Korea) for 20 min to eradicate all vegetative cells. Cell suspensions in the tubes were 10-fold serially diluted in PW, and then 0.1 mL of undiluted cell suspensions or diluents were spread plated onto mannitol–egg yolk–polymyxin agar (Difco) to enumerate *B. cereus*. When low bacterial numbers were anticipated, 250 μL of undiluted cell suspension was plated onto four plates of media. The plates were incubated at 37°C for 24 h, and colonies were counted.

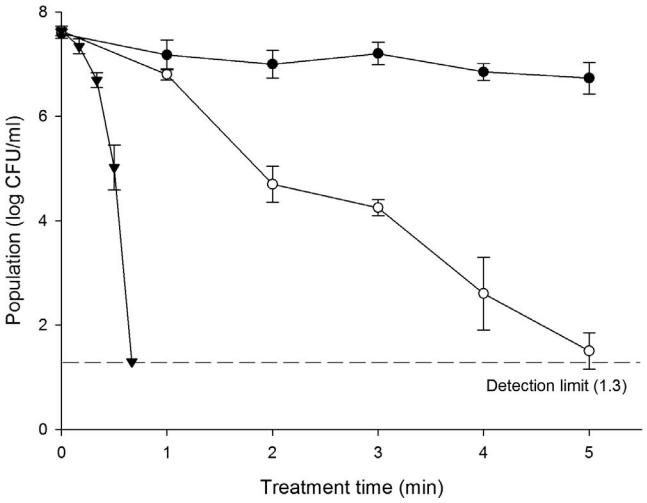

FIGURE 1. *Populations of* Bacillus cereus *spores (log CFU per milliliter) subjected to 200°C (●), 250°C (○), and 300°C (▼) SHS treatment.*

DPA release measurement. Untreated or treated spore-cell suspensions were prepared as described previously, except that distilled water was used instead of PW. The DPA release levels of untreated and treated *B. cereus* spores were determined with fluorescence emitted by the binding of terbium ions (Tb^{3+}) to DPA *(10)*. One milliliter of untreated or treated cell suspension was combined with terbium chloride ($TbCl_3$; Sigma-Aldrich) to yield 50 μM *(25),* and fluorescence was measured with a spectrofluorophotometer (SpectraMax M2e; Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 270 nm and an emission wavelength of 545 nm. Untreated samples and autoclaved (121°C for 15 min) samples were used as negative and positive controls, respectively, and the following equation was used to calculate the DPA release ratio (%):

$$\text{DPA release ratio } (\%) = (F_T - F_N)/(F_P - F_N)$$

where F_T, F_N, and F_P are the fluorescence values of treated, untreated (negative control), and autoclaved (positive control) samples, respectively.

Statistical analysis. All inactivation experiments were replicated three times. The analysis of variance procedure of the SAS (version 9.3, SAS Institute Inc., Cary, NC) was used to analyze the replicated data. Duncan's multiple range test was used to separate calculated mean values. A 95% significance level ($P = 0.05$) was used to determine significant differences.

RESULTS AND DISCUSSION

Increasing the temperature of SHS was an effective way to inactivate *B. cereus* on stainless steel (Fig. 1). The application of 200°C SHS on stainless steel resulted in a slight reduction (<1 log CFU) of *B. cereus,* whereas a more than 5-log reduction in *B. cereus* level was achieved by applying 250 and 300°C SHS for 4 min and 40 s, respectively. The same tendency was observed in Zhou et al. *(26),* who reported that peak temperature is considerably important to inactivate bacterial spores, while heating time has a minor impact. This phenomenon also can be understood by the Arrhenius equation, which indicates that treatment temperature and time are critical parameters for

TABLE 1. *Reduction of* B. cereus *spores subjected to 250°C superheated steam for 1 min (SHS), UV-C irradiation for 15, 30, and 60 min alone, or sequentially treated with 1 min of 250°C SHS before UV-C (SHS+UV-C) or after UV-C (UV-C+SHS)*[a]

	UV-C treatment time (min):		
	15	30	60
SHS	0.68 ± 0.27 A a[b]	0.68 ± 0.27 A a	0.68 ± 0.27 A a
UV-C	1.06 ± 0.40 A a	1.18 ± 0.26 A a	1.68 ± 0.23 B a
UV-C+SHS	2.30 ± 0.50 B a	2.48 ± 0.60 B a	2.98 ± 0.59 C a
SHS+UV-C	2.73 ± 0.92 B a	2.70 ± 0.61 B a	3.06 ± 0.51 C a

[a] Mean log CFU values ± standard deviations.
[b] Values in the same column with the same uppercase letter are not significantly different ($P > 0.05$). Values in the same row with the same lowercase letter are not significantly different ($P > 0.05$).

cell destruction. Several technologies have been used to inactivate bacterial spores inoculated on food contact surfaces. Nam et al. *(13)* used gaseous ClO_2 treatment to inactivate *B. cereus* spores attached to stainless steel coupons and reported that 1 and 6 h were needed to inactivate planktonic spores and spores in biofilms, respectively. Udompijitkul et al. *(23)* reported that inducing germination of *Clostridium perfringens* spores increases the efficacy of surface disinfectants. Although these studies on the inactivation of bacterial spores on food contact surfaces were reported, to our knowledge, our study is the first attempt to inactivate *B. cereus* spores inoculated on stainless steel by using SHS.

The application of 250°C SHS treatment for 1 min was not sufficient to thoroughly inactivate *B. cereus* spores. Therefore, inoculated stainless steel was subjected to UV-C irradiation before or after SHS to increase the inactivation efficacy. Combined treatment with UV-C irradiation and SHS showed a synergistic effect on the inactivation of *B. cereus* spores regardless of treatment sequence (Table 1). For example, reduction in the *B. cereus* level by 1 min of 250°C SHS + 1 h of UV-C radiation treatment was 3.06 log CFU, which was significantly higher than the sum of each treatment (0.68 + 1.68 = 2.36 log CFU). Increased treatment time from 15 to 60 min did not have a significant effect on the reduction of *B. cereus* spores by UV-C irradiation or combination treatments ($P > 0.05$). The synergistic bactericidal effects of heat with UV-C treatment and its mechanism have been widely reported *(5, 7),* but most of the studies focused on vegetative cell inactivation rather than spore inactivation because mild heat was used. Moreover, previously reported hurdle technologies, such as heat plus pressure *(24)* and high pressure plus nisin *(2)* combination treatments, are not applicable to the inactivation of spores on food contact surfaces. In this regard, the sequential treatment of SHS and UV-C irradiation used in the present study is a novel hurdle technology to inactivate *B. cereus* spores inoculated on stainless steel. Moreover, decreasing the target temperature of SHS from 300 to 250°C in combination with UV-C irradiation would reduce total energy consumption, considering that SHS is high-energy–consuming equipment.

TABLE 2. *Dipicolinic acid (DPA) release ratios of* Bacillus cereus *spores subjected to 1 min of 250°C superheated steam (SHS), 15 min of UV-C irradiation, sequential treatment of UV-C after SHS (SHS+UV-C), and SHS after UV-C (UV-C+SHS)*[a]

	DPA (%)
SHS	9.25 ± 6.50 A[b]
UV-C	40.8 ± 10.1 B
SHS+UV-C	67.3 ± 16.1 C
UV-C+SHS	71.7 ± 17.4 C

[a] Mean values ± standard deviations.
[b] Values in the same column with the same uppercase letter are not significantly different ($P > 0.05$).

Inactivation efficacy by combination treatment was confirmed with DPA release ratios (percentage). Because increasing the UV-C treatment time had no significant effect ($P > 0.05$) for the combination treatment, the DPA release ratio was identified with 1 min of 250°C SHS, 15 min of UV-C, and combination treatments. A synergistic effect was observed from the DPA release ratio of *B. cereus* spores after combining SHS with UV-C irradiation treatments. The DPA release ratios of *B. cereus* spores were 9.25% ± 6.50%, 40.8% ± 10.1%, 67.3% ± 16.1%, and 71.7% ± 17.4% by SHS, UV-C, SHS+UV-C, and UV-C+SHS treatments, respectively (Table 2). It is well known that DPA has a significant role in the heat or UV-C resistance of *Bacillus* spores. Previous research has indicated that DPA is responsible for the high resistance of *Bacillus* spores to dry or wet heat treatment *(16)* and that DPA-deficient *Bacillus* spores are more susceptible to UV-C irradiation than their DPA-containing counterparts *(21)*. In this regard, an increased DPA release ratio by a combination treatment of SHS and UV-C has a significant role in this synergistic bactericidal effect.

In conclusion, *B. cereus* spores inoculated on stainless steel were inactivated with SHS or a combination treatment of SHS and UV-C radiation in the present study. When SHS treatment was applied alone, temperatures higher than 250°C were needed to eliminate the *B. cereus* spores within 5 min. A combination treatment of 250°C SHS with UV-C irradiation showed a synergistic effect on the inactivation of *B. cereus* spores, and the synergistic bactericidal mechanism was determined with DPA release ratios. The treatment sequence had no significant effect on the inactivation efficacy or DPA release ratio. These results indicate that the combined treatment of SHS and UV-C irradiation can be used effectively to inactivate *B. cereus* spores on stainless steel. The hurdle technology used in the present study can be applied to ensure microbiological safety in food processing facilities, especially for clean-in-place processes.

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